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Implementation of a Non-lethal Biopsy Punch Monitoring Program for Mercury in Smallmouth Bass, *Micropterus dolomieu* Lacepède, from the Eleven Point River, Missouri

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Abstract A non-lethal biopsy method for monitoring mercury (Hg) concentrations in smallmouth bass (*Micropterus dolomieu*; smallmouth) from the Eleven Point River in southern Missouri USA was evaluated. A biopsy punch was used to remove a muscle tissue plug from the area immediately below the anterior dorsal fin of 31 smallmouth. An additional 35 smallmouth (controls) were held identically except that no tissue plug was removed. After sampling, all fish were held in a concrete hatchery raceway for 6 weeks. Mean survival at the end of the holding period was 97 % for both groups. Smallmouth length, weight and Fulton's condition factor at the end of the holding period were also similar between plugged and non-plugged controls, indicating that the biopsy procedure had minimal impact on growth under these conditions. Tissue plug Hg concentrations were similar to smallmouth Hg data obtained in previous years by removing the entire fillet for analysis.

Keywords Mercury · Smallmouth bass · *Micropterus dolomieu* · Biopsy punch · Survival

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Mercury (Hg) is a ubiquitous pollutant released to the environment from natural and anthropogenic sources (US EPA 2001). Although much of the Hg in aquatic ecosystems originates from the atmosphere as inorganic Hg, biogeochemical processes convert inorganic Hg to methylmercury (MeHg; Wiener et al. 2002; Sanheinrich and Wiener 2011). Most (>90 %) of the Hg in fish is MeHg, which is highly toxic and biomagnifies in aquatic food webs (Neumann and Ward 1999; Wiener et al. 2002). MeHg is therefore widely recognized as a threat to wildlife and human health (Wiener et al. 2002), as evidenced by nationwide human fish consumption advisories for Hg (US EPA 1997, 2001, 2004) and protective toxicity criteria for wildlife (US EPA 1997).

Black bass (*Micropterus* spp.) in Missouri through 2011 had fillet Hg concentrations ranging from 8.0 to 1,300 ng/g wet weight (ww; MDC 2013), with 40 % of 693 samples exceeding the US EPA recommended human health criterion of 300 ng/g ww (US EPA 2001). The Missouri Department of Health and Senior Services (MDHSS) has reviewed these data against its own risk criteria and recommends that sensitive populations (pregnant women, women of childbearing age, nursing mothers, and children <13 years old) consume not more than one meal per month of largemouth bass (*M. salmoides*) or smallmouth bass (*M. dolomieu*; smallmouth) greater than 12 inches (305 mm) total length (TL) from Missouri waters (MDHSS 2013). Given the importance of the black bass fishery in Missouri, continued monitoring will be required to maintain accurate fish consumption advisories and to determine trends.

The Missouri Department of Conservation (MDC) manages several high-value smallmouth fisheries for which the public has expressed concerns about excessive fish removal for study but where there are also public health concerns due to elevated Hg concentrations. One example

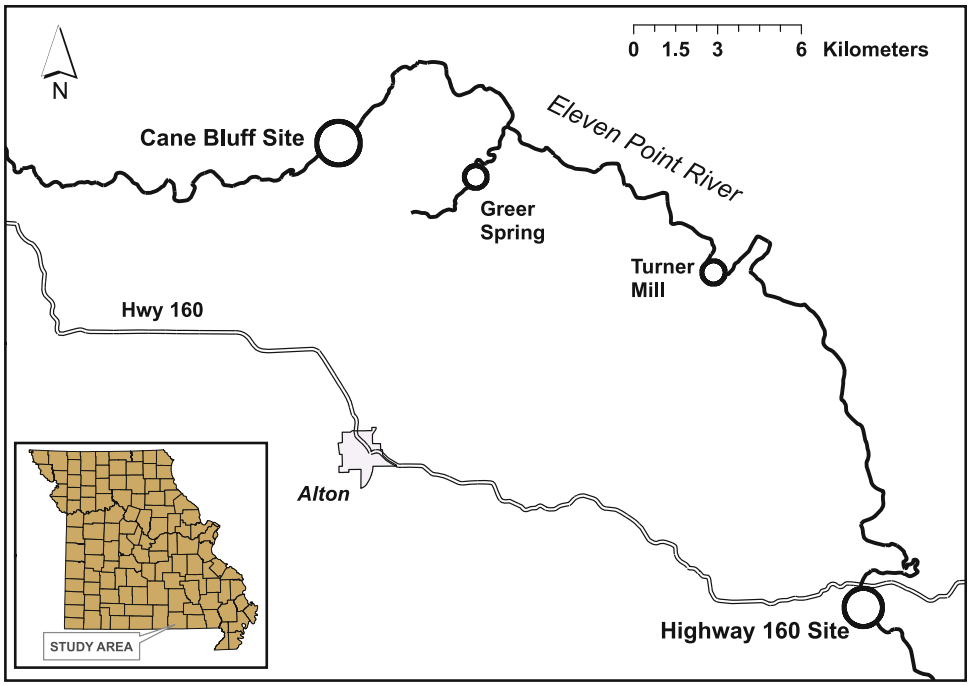

Fig. 1 Map of the study area showing collection sites at Cane Bluff and Highway 160 on the Eleven Point River in Oregon County, Missouri along with Greer Spring and Turner's Mill

Table 1 Summary of key information from fish studies using the biopsy punch to monitor total mercury (Hg), methylmercury (MeHg), and selenium (Se)

Investigation	Species[a]	Survival assessed	Growth assessed	Contaminants monitored
Lockhart et al. (1972)	Northern pike	Yes	No	MeHg
Freeman and Horne (1973)	Swordfish	No	No	Hg
Friedmann et al. (1996)	Northern pike	No	No	Hg
Tyus et al. (1999)	Bonytail chub, razorback suckers, rainbow trout	Yes	Yes	None
Osmundson et al. (2000)	Colorado pikeminnow	Yes	No	Se
Hamilton et al. (2002)	Razorback sucker	Yes	No	Se
Cizdziel et al. (2002a)	Striped bass, channel catfish, largemouth bass, blue tilapia, bluegill, rainbow trout	No	No	Hg
Cizdziel et al. (2002b, 2003)	Striped bass, channel catfish, largemouth bass, blue tilapia, bluegill, rainbow trout	No	No	Hg
Baker et al. (2004)	Lake whitefish, northern pike	Yes	No	Hg
Hamilton et al. (2004)	Colorado pikeminnow	No	No	Se
Peterson et al. (2005)	13 Freshwater spp.	No	No	Hg
Schmitt and Brumbaugh (2007)	Smallmouth bass	No	No	Hg
Burger and Gotchfeld (2011, 2012)	19 Saltwater spp.	No	No	Hg, Se
Ackerson et al. (This study)	Smallmouth bass	Yes	Yes	Hg

[a] See text for the scientific name of species listed

is the trophy smallmouth fishery of the Eleven Point River (EPR) in south-central Missouri (Fig. 1), where Hg concentrations have historically exceeded the US EPA criterion in a significantly greater proportion of samples compared to statewide trends (i.e., 64 % of samples exceed criterion in EPR vs. 40 % statewide). In addition to the fish consumption advisory recommendation for smallmouth, the exceedance of the EPA criterion resulted in EPR being added to Missouri's list of impaired waters in 2002 as required under Section 303(d) of the Clean Water Act (MDNR 2011). This designation will necessitate even more frequent and continued monitoring of EPR smallmouth.

Researchers have proposed several non-lethal biopsy sampling methods to monitor fish Hg in lieu of euthanasia

Table 2 Size classes and disposition of smallmouth from two sites on the Eleven Point River (Cane Bluff and Highway 160)

Size class mm (inches)	Cane Bluff (19 fish)		Highway 160 (47 fish)	
	Number control fish	Number plugged fish	Number control fish	Number plugged fish
279–305 (11–12)	2	3	6	4
306–330 (12–13)	3	1	4	4
331–356 (13–14)	2	2	3	5
357–381 (14–15)	1	1	9	4
382–406 (15–16)	1	3	4	4

and fillet removal (Schmitt and Brumbaugh 2007 and references cited therein). Among these, the biopsy punch has received considerable attention by researchers but no survival information is available for smallmouth (Table 1). The purpose of this study was to evaluate survival and general health of smallmouth following tissue plug removal under field conditions for the purpose of Hg monitoring.

Materials and Methods

Much of the EPR in Missouri (Fig. 1) is within the Mark Twain National Forest and is part of the National Scenic River system. Greer Spring, a large conduit spring that feeds the EPR, supplies cold water (typically 12–15°C) year-round and more than doubles the base flow (Miller and Wilkerson 2000). As a result, the EPR supports cold- and cool-water species (i.e., percids and salmonids) for about 20 mi (32 km) downstream from Greer Spring. Greer Spring input effectively separates the EPR into two reaches with distinctly different temperature regimes. Smallmouth occur in both reaches of the EPR, so fish from each reach were sampled to determine if the temperature difference might affect post-sampling fish survival (i.e., post-sampling infections might be higher at higher acclimation temperatures). Cane Bluff is upstream of the confluence of Greer Spring (Fig. 1); temperatures in late summer typically average 20–23°C (Miller and Wilkerson 2000). At Highway 160 (Hwy 160), which is below Greer Spring (Fig. 1), late summer temperatures are typically 16–19°C (Miller and Wilkerson 2000). Base flow at Hwy 160 is more than twice the flow at Cane Bluff due to the influence of Greer Spring. Based on previous sampling experience from these two reaches, more fish were targeted for collection at Hwy 160 ($n = 40$) than at Cane Bluff ($n = 20$).

Smallmouth were collected by electrofishing and held in recirculating tanks. A wide size range was sought at both sites to facilitate site-specific statistical analysis of fish size:Hg concentration relations. Fish were measured (TL) to ensure that similar size ranges were obtained at both sites. The fish were transported in an aerated tank truck to a nearby hatchery for sampling and observation. At the hatchery, half the fish from each site were randomly selected for biopsy sampling, with the rest reserved as controls. Individual fish were not tagged or otherwise marked for identification to avoid additional external lesions or stress. All field procedures conformed with recommendations of the American Fisheries Society (AFS), American Institute of Fishery Research Biologists, and the American Society of Ichthyologists and Herpetologists (AFS et al. 2004); and with requirements of the US Laboratory Animal Welfare Act, the Interagency Research Animal Committee, and all USGS guidelines for the humane treatment of test organisms during culture and experimentation. Use of trade, product, or firm names is for descriptive purposes and does not constitute endorsement.

Biopsy samples were collected as described by Schmitt and Brumbaugh (2007) and demonstrated in a training video (Electronic Supplementary Material 2013). Briefly, fish were retrieved from the holding tank, measured (TL), wrapped in a tared wet cloth, and weighed. The wrapped fish were placed on a v-board to further reduce movement. A small section of the wetted towel was pulled back to expose an area just below the dorsal fin. Scales were gently removed from the area on one side of the fish using a knife. A tissue plug sample was obtained from the dorsal musculature beneath the anterior dorsal fin with a 5-mm (dia) disposable biopsy punch (Uni-Punch Premier Disposable Biopsy Punch, Delasco, Council Bluffs, IA). The wounds were not sutured or glued closed after sampling in order to maintain a simple field protocol and to minimize the time the fish were out of water. [Note: contrary to some reports, the use of cyanoacrylate surgical adhesives on fish may lead to tissue necrosis or dehiscence (Wagner et al. 2011)]. The tissue plug was extracted from the biopsy punch with stainless-steel forceps and placed on aluminum foil. The skin was cut from the exterior surface with a stainless steel scalpel, and the sample was transferred to a pre-cleaned polyethylene vial. Between samples all instruments and work surfaces were thoroughly cleaned or replaced to prevent external and cross-contamination. Samples were placed on ice immediately after collection and, after all samples were collected, frozen (−20°C) until thawed for analysis.

After sampling the fish were placed in an aerated holding tank for observation, then transferred to outdoor concrete raceways (8/29/2007). Fish from each location were held separately and fed daily with live goldfish (*Carassius auratus*); water temperature was 15–16°C. Fish were held at the hatchery for 6 weeks and observed daily for survival, abnormal behavior, or other signs of stress. At

the end of the observation period, each fish was examined, re-measured, and re-weighed. All surviving fish were then released at their respective collection sites.

Biopsy samples were analyzed as described by Schmitt and Brumbaugh (2007) and May et al. (2009). Briefly, the samples were lyophilized using a vacuum desiccator and analyzed for total Hg by combustion amalgamation atomic absorption spectrophotometry (CAAAS; US EPA method 7473). Dry-weight concentrations were converted to and reported as wet weight concentrations using a mean moisture content for smallmouth muscle of 78 % (n = 62; Schmitt and Brumbaugh 2007). Because most of the Hg in fish muscle is MeHg, measurement of total Hg is effectively a measure of MeHg (Sanheinrich and Wiener 2011). Precision among replicate plugs from the same smallmouth collected using our procedure has been shown to be high (RSD = 2.2 %–2.4 % in 5 sets of triplicates; Schmitt and Brumbaugh 2007). Therefore, only one plug was obtained from each fish to minimize stress to the fish. Quality assurance (QA) measures included the analysis of blanks, instrument calibration standards, and certified reference materials. Method limits-of-detection were 18–23 ng/g dry weight, which were exceeded in all samples. Overall, the QA results indicated an acceptable level of precision and accuracy and no evidence of sample contamination. Additional QA information is available elsewhere (May et al. 2009).

Fulton's condition factor (K) was calculated using length and weight data obtained at the time of the tissue biopsy and after the 6 week holding period as follows: $K = (W/TL^3)*100$, where W is fresh weight (g) and TL is in cm (Nash et al. 2006). Summary statistics (mean and standard deviation) were calculated for these parameters but before-and-after statistical analyses were not performed because individual fish were not marked (i.e., samples were not independent). Schmitt and Brumbaugh (2007) analyzed tissue plug and fillet samples from individual smallmouth (n = 12) collected at Turner's Mill, which is in the cold-water reach of the EPR between Greer Spring and Hwy 160 (Fig. 1). We included these data in our analyses for comparison. All statistical analyses were conducted using Version 9.2 of the Statistical Analysis System (SAS Institute, Carey, NC). Descriptive statistics (mean, range and other distributional information) for each group of fish were examined. Relations between and among variables were examined with ANOVA, ANCOVA, and linear regression. Preliminary analyses indicated that the Hg:fish size relations differed between sites. Hg concentrations were therefore normalized for fish size with ANCOVA by adjusting to the grand mean TL (335 mm) using site-specific linear regressions (Schmitt et al. 2011). Statistical significance testing was set at $p < 0.05$ unless otherwise indicated.

Results and Discussion

A total of 66 smallmouth (19 from Cane Bluff, 47 from Hwy 160) ranging in size from 279 to 406 mm TL were collected on August 28, 2007; all size classes were well represented among the sample groups (Table 2). Biopsy samples for Hg analysis were obtained for 10 fish from Cane Bluff and 21 fish from Hwy 160 on August 29, 2007 and all fish were held in outside raceways until October 10, 2007. Two smallmouth died (one plugged fish from Cane Bluff on September 8 was 396 mm and one control from Hwy 160 on October 10 was 396 mm) during the holding period (Table 3). Necropsies were not performed; however, no lesions, infections or other obvious potential causes for the mortalities were evident. Survival was 90 % for the plugged fish from Cane Bluff and 100 % for control fish, and there was no apparent relation between fish size and survival. At Hwy 160, survival was 100 % for the plugged fish and 96 % for controls. Overall survival (both sites) was 97 % for plugged fish and 97 % for controls, indicating that the plug removal did not affect survival within this timeframe and conditions. Visual examination of the wounds at the end of the holding period indicated that scales were forming a protective layer over the wound and only a slight indentation remained where the sample had been removed (Fig. 2).

Table 3 Length, weight, Fulton's condition factor (K) and survival in control and treated (tissue plug removed) smallmouth from two sites on the Eleven Point River (Cane Bluff and Highway 160)

Parameter	Cane Bluff		Highway 160	
	Control fish	Plugged fish	Control fish	Plugged fish
Number fish at start	9	10	26	21
Length initial, cm[a]	34.0 (3.5)	33.8 (3.9)	34.6 (3.5)	34.5 (3.6)
Length at week 6, cm[a]	33.4 (3.2)	33.5 (3.3)	34.4 (3.5)	34.3 (3.4)
Length increase, %[b]	−1.8	−0.9	−0.6	−0.6
Weight initial, g[a]	499 (186)	487 (225)	566 (174)	551 (177)
Weight at week 6, g[a]	580 (193)	568 (162)	600 (185)	590 (186)
Weight gain, %[b]	16.2	16.6	6.0	7.1
K initial[a]	1.22 (0.10)	1.19 (0.14)	1.32 (0.08)	1.30 (0.10)
K at week 6[a]	1.52 (0.15)	1.48 (0.14)	1.43 (0.09)	1.42 (0.09)
K increase, %[b]	24.6	24.4	8.3	9.2
Survival at week 6, #	9	9	25	21
Survival at week 6, %	100	90	96	100

[a] Mean (standard deviation)

[b] ((week 6 value-initial value)/initial value)*100

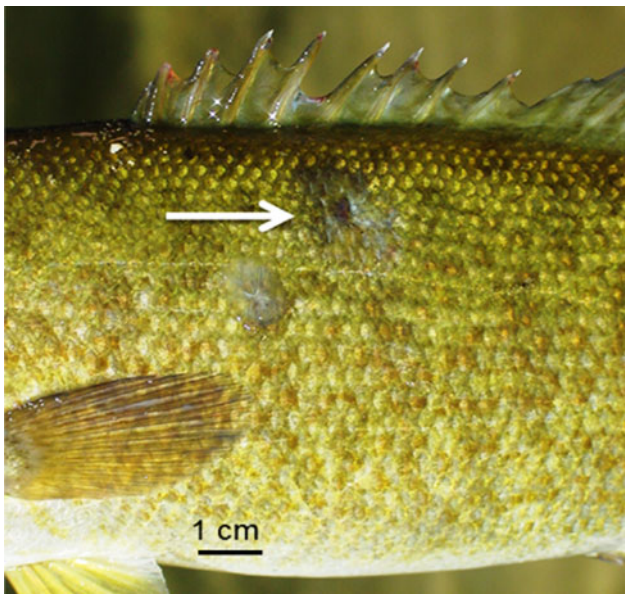

Fig. 2 Smallmouth bass sampling wound from the biopsy punch (*arrow*) after 6 weeks

Despite the growing acceptance of biopsy sampling for Hg and Se, there have been few post-sampling assessments of survival (Table 1). Tyus et al. (1999) found no effects on survival in the laboratory of bonytail chub (*Gila elegans*), razorback sucker (*Xyrauchen texanus*), or rainbow trout (*Oncorhycnhus mykiss*) held for 3, 4 and 6 months, respectively, following tissue plug removal. Repeated removal of tissue plugs from individual razorback suckers held in holding ponds did not affect survival compared to controls (Hamilton et al. 2002). Baker et al. (2004) found no difference in recapture probabilities of northern pike (*Esox lucius*) with or without tissue plug removal. Survival was implicit for wild Colorado pikeminnow (*Ptychocheilus lucius*) on which scars from repeated biopsy punch sampling were observed (Osmundson et al. 2000) and in northern pike re-sampled after having been transported between lakes (Lockhart et al. 1972). In our study, smallmouth survival was not adversely affected by collection, handling, holding, or plug removal, which is consistent with previously reported results for other species.

Smallmouth growth over the 6-week holding period did not appear to be affected by tissue plug removal. Percent increase in fish length during the holding period was slightly negative (but near zero) with no apparent effect of tissue plug removal (Table 3). Percent weight gain was positive in all groups during the holding period with similar values for control and plugged fish from both sites; however, Cane Bluff fish gained substantially more weight than fish from Hwy 160 (Table 3). Percent increase in K, which incorporates both length and weight measurements, followed a similar pattern to weight gain (Table 3). An absence of fish growth effects following tissue plug removal was also observed by Tyus et al. (1999) for bonytail chub, razorback sucker, and rainbow trout held in the laboratory. There was no apparent relation between fish size and growth rate in any of the groups. Although our growth data could not be statistically compared because individual fish were not tagged, these data and previous reports indicate that tissue plug removal is not likely to substantially affect fish growth.

Table 4 Regression parameters for Hg concentrations versus fish length and mean Hg concentrations in smallmouth bass collected from the Eleven Point River at Cane Bluff and Highway 160 in 2007 and from Turner's Mill in 2005 (from Schmitt and Brumbaugh 2007)

Site	Regression parameter[a]				Hg concentration, ng/g ww	
	n	Intercept	Slope	R^2	Mean (adjusted[b])	Mean (unadjusted)
Cane Bluff	10	**−1.76**	**1.73**	**0.45**	449[a]	450
Highway 160	21	**−0.32**	**1.11**	**0.22**	325[b]	326
Turner's Mill	12	0.25	0.87	0.17	276[b]	260

[a] Values in bold differ significantly from 0 ($p < 0.05$)

[b] Mean adjusted to grand mean total length (335 mm) by ANCOVA. Adjusted means followed by the same letter are not significantly different ($p < 0.05$)

Mean Hg concentration, adjusted for fish length, was significantly greater at Cane Bluff than at Hwy 160 (Table 4). Slopes (fish length vs. Hg) and intercepts from both sites were significantly different from zero. The slope estimate for fish from Cane Bluff appeared greater than at Hwy 160 (Table 4), indicating more rapid Hg bioaccumulation at Cane Bluff. Although the Turner's Mill slope was not statistically significant, it was similar to the Highway 160 slope (Table 4). Turner's Mill has a similar temperature profile as Hwy 160. Adjusted mean Hg for fish from Turner's Mill was not different from Hwy 160, but both were lower than Cane Bluff (Table 4).

In addition to a greater Hg concentration and slope at Cane Bluff compared to Hwy 160, weight gain and K were also notably greater at Cane Bluff (Table 3). Cane Bluff is above Greer Spring, and the EPR in this area is on average about 4°C warmer in summer than at Hwy 160. Although temperature differences between the sites may at least partly explain the difference in growth and Hg bioaccumulation, the precise explanation is unknown and beyond the scope of this study. Regardless, our results clearly indicate that the difference in growth between the sites is not related to the removal of the tissue plug.

Morizot et al. (1990) identified six criteria that should be met before implementing a minimally invasive procedure

in fish field studies. Following is our assessment of the adequacy of current information for each criterion relative to the use of the biopsy punch for monitoring contaminants in fish:

- Criterion 1 (minimum impact on fish survival) is met based on the survival data summarized in this paper. Collectively, our data indicate that a field implemented program using a small biopsy tissue punch (e.g., ≤5 mm) will have minimal impact on fish survival.
- Criterion 2 (minimal impact on fish health and fitness) is met based on the observation that no adverse effects on growth or condition (K) were observed and that the fish appeared to be in good health at the time of release. We believe that current data provide sufficient information to move forward with the use of this procedure in field studies while also recognizing that more information on health and fitness for smallmouth and other species should be obtained where possible.
- Criterion 3 (sufficient tissue mass) is met for Hg analysis of the biopsy punch tissue plug by CAAAS. The 5 mm biopsy punch method has also been shown to provide sufficient tissue for monitoring Se (Waddell and May 1995; Osmundson et al. 2000; Hamilton et al. 2004; Burger and Gotchfeld 2011, 2012). Biopsy samples from black bass of "catchable" size (>250 mm TL) typically yield 40–100 mg of wet tissue, or about 10–20 mg dry. Analysis by CAAAS consumes most of this material. Considerably more tissue (gram quantities) would be required to support the analysis of other routinely monitored contaminants such as organochlorine pesticides and polychlorinated biphenyls using currently available methods. Consequently, biopsy sampling does not presently seem to be a feasible alternative to traditional (i.e., fillet or whole-fish) sampling for multi-contaminant monitoring.
- Criterion 4 (minimal post-sampling treatment of fish and easy storage under laboratory and field conditions) is met. We used no post-sampling treatment and the storage of field collected plug sample on ice and then in the laboratory freezer is well within the capability of field contaminant monitoring programs.
- Criterion 5 (applicable for most fish taxa) is met for many, but not all fish taxa. For the muscle biopsy procedure to be used for monitoring in a particular fish species, it is necessary to evaluate the representativeness of the contaminant concentration in the tissue subsample relative to the entire fillet, which could differ among species. Schmitt and Brumbaugh (2007) previously validated the tissue plug method for smallmouth by demonstrating that the Hg concentrations in tissue plugs and in fillets from the same fish were identical ($r = 0.99$, $p < 0.01$, n = 59). Other freshwater species for which the method has been validated for Hg include largemouth bass, lake whitefish (*Coregonus clupeaformis*), northern pike, striped bass (*Morone saxatilits*), bluegill (*Lepomis macrochirus*), rainbow trout, channel catfish (*Ictalurus punctatus*), blue tilapia (*Oreochromis aureus*), and northern pikeminnow (*Ptychocheilus oregonensis*; Cizdziel et al. 2002b; Baker et al. 2004). Validation information for Se is available for the species listed in Table 1.
- Criterion 6 (minimal training required for technical personnel) is met based on the following observations. To minimize the amount of "hands-on" training of field staff, we kept the procedure simple and prepared a written step-by-step procedure containing a list of materials. We also created a training video that is available on-line for review prior to using the procedure (Electronic Supplementary Material 2013). As part of this study, we had field biologists perform all aspects of the process, after which they indicated that they were confident in their ability to conduct the procedure without supervision. Subsequent to this study, MDC implemented the biopsy punch method for Hg monitoring statewide. Field biologists have indicated that a written description of the procedure together with the training video is sufficient for implementation of the method (Personal Communication). We note, however, that laboratories also need to be (or become) familiar with the analysis of the small samples.

Based on this assessment, we feel the biopsy punch meets all of the Morizot et al. (1990) criteria for Hg and Se and for several fish species. Tissue plug sampling with a biopsy punch represents a viable alternative to euthanizing fish for determining fillet Hg concentrations. Information presented here indicates that a tissue-plug based Hg monitoring program for smallmouth in the EPR and elsewhere can be implemented with minimal impacts on fish survival and growth. Further documentation of long-term fitness and survival under controlled conditions, wherein fish could be individually marked and analyzed repeatedly, would be useful, as would the development and testing of non-lethal protocols for multi-contaminant monitoring.

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